LESSON 4:
STERILIZATION TECHNIQUES

Objective

The most important and rather difficult aspect of the in vitro techniques is the requirement to carry out various operations under aseptic conditions. Bacteria and fungi are the most common contaminants observed in cultures. You will be surprised to know that they grow much faster than the cultured tissue and may also give out metabolic wastes which are toxic to plant tissues. Therefore, sterilization is essential.

Sterilization of Culture Vessels and Instruments

1. Glasswares, metal instruments and aluminium foil can be sterilized by exposure to hot dry air (160°C – 180°C) for 2-4 hr. in a hot- air oven. All items should be properly sealed before sterilizing.
2. Autoclaving is a method of sterilizing with water vapour under high pressure. Nearly all microbes are killed on exposure to the superheated steam of an autoclave. Normally, glasswares, cotton plugs, gauze, plastic caps, filters or pipettes are autoclaved at 121oC and 15 psi for 15-20 min. Some types of plastic labware can also be repeatedly autoclaved.
3. Flame Sterilization: Metallic instruments like forceps, scalpels, needles and spatulas are sterilized by dipping in 95% ethanol, followed by flaming and cooling.This technique is called flame sterilization. Autoclaving of metallic instruments is generally avoided as they may rust and become blunt.
4. Dry Sterilization: Now a days, in place of flame sterilization, dry sterilization of instruments using 'steri- pots' is practised in order to avoid instant fires caused by alcohol.
   STERIIZATION OF NUTRIENT MEDIA:
5. Autoclving: Culture media in glass containers is sealed with cotton plugs, aluminium foils or plastic closures and autoclaved at 15 psi and 121°C for 15-40 min. from the time the medium reaches the required temperature. Exposure time depends on the volume of the liquid to be sterilized (see Box 1). The pressure should not exceed 20 psi, as higher pressure may lead to decomposition of carbohydrates and other components of a medium.
6. Filter Sterilization: Vitamins, amino acids, plant extracts, hormones and carbohydrates are 'thermolabile' and may decompose during autoclaving. So, they are filter sterilized where the solutions are passed through a bacteria proof membrane- filter under positive pressure. A 'Millipore' or 'Seitz filter' with a pore size of not more than 0.2 um is generally used in filter sterilization.

Box 1. Plant tissue culture media are generally sterilized by autoclaving at 121 °C and 1.05 kg/cm^2 (15-20 psi). The time required for sterilization depends upon the volume of medium in the vessel. The minimum times required for sterilization of different volumes of medium are listed below. It is advisable to dispense medium in small aliquots whenever possible as many media components are broken down on prolonged exposure to heat. There is evidence that medium exposed to temperatures in excess of 121 °C may not properly gel or may result in poor cell growth.

Minimum Autoclaving Time Forplant Tissue Culture Medium

Volume of Medium per Vessel (ml)	Minimum Autoclaving (min)*
25	20
50	25
100	28
250	31
500	35
1000	40
2000	48
4000	63

*Minimum autoclaving time includes the time required for the liquid volume to reach the sterilizing temperature(121 °C) and 15 min. at 121 °C (Burger, 1988). Times may vary due to differences in autoclaves. Validation with your system is recommended.

Several medium components are considered thermolabile and should not be autoclaved. Stock solutions of the heat labile components are prepared and filter sterilized through a 0.22 µm filter into a sterile container. The filtered solution is aseptically added to the culture medium, which has been autoclaved and allowed to cool to approximately 35-45 °C. The medium is then dispensed under sterile conditions. Experimentation with your system is recommended.

Sterilization of Culture Rooms and Transfer Area:

Initially, the culture rooms are cleaned by gently washing all floors and walls with a detergent soap. This is followed by carefully wiping then with 2% sodium hypochlorite solution or 70% ethyl alcohol.

The transfer area is also sterilized once or twice a month by washing with a commercial brand of antifungal spirocyte. Larger transfer rooms are best sterilized by exposure to UV light, when there are no experiments in progress (since UV is harmful to eyes). HEPA filter ventilation unit can also be installed in the room. Laminar air-flow hoods are usually sterilized by switching on the hood and wiping the working surface with 70%ethyl alcohol, 15 min. before starting any operation under the hood.

Aseptic Culture Technique

Sterilization of Plant Material: (see Box 2)

During sterilization of plant tissues, the living materials should not lose their biological activity and only bacterial or fungal contaminants should be eliminated. Plant organs or tissues are therefore, only surface-sterilized by treatment with a disinfectant solution at suitable concentrations for a specified period. The disinfectants most widely used are:

i. Mercuric chloride (0.1 – 1.0%) for 2-10 min,

ii. Bromine water (1-2%), for 2-10 min,

iii. Silver nitrate (1%), for 5-30 min.

iv. Sodium hypochlorite (0.5-5%), for 5-30 min.

Addition of a few drops of a surfactant (triton–x or tween-80) to the solution before exposing to sterilant may increase sterilization efficiency.

Box 2 Explant Sterilization

1. Wash explants in a mild detergent before treatment with the disinfecting solution. (Herbaceous material may not require this step).
2. Rinse explants thoroughly under running tap water for 10-30 minutes.
3. Submerge explants into the disinfectant solution. Seal bottle and gently agitate.
4. Under sterile conditions, decant the solution and rinse explants several times with sterile distilled water.

Commonly Used Disinfectantsfor Plant Tissue Culture

Disinfectant	Product No.	Concentration (%)	Exposure (min)
Calcium hypochlorite	21,138-9	9-10	5-30
Sodium hypochlorite*	42,504-4	0.5-5	5-30
Hydrogen peroxide	H 1009	3-12	5-15
Ethyl alcohol	E7148	70-95	0.1-5.0
Silver nitrate	S 7276	1	5-30
Mercuric chloride	M 1136	0.1-1.0	2-10
Benzalkonium chloride	B 1383	0.01-0.1	5-20

*Commercial bleach contains about 5% sodium hypochlorite, and thus may be used at a concentration of 10-20%, which is equivalent to 0.5-1.0% sodium hypochlorite.

Sterilization procedures may be enhanced by:

1. Placing the material in a 70% ethyl alcohol solution prior to treatment with another disinfectant solution. The use of a two-step (two-source) sterilization procedure has proven beneficial with certain species.
2. Using a wetting agent, such as Tween 20 or 80 can be added to the disinfectants to reduce surface tension and allow better surface contact.
3. Conducting the sterilization process under vacuum. This results in the removal of air bubbles and provides a more efficient sterilization process.

Orchid Seed Sterilization

Orchid seeds are very small and contain little to no food reserves. A single seed capsule may contain 1,500 to 3,000,000 seeds. Sowing the seed in vitro makes it possible to germinate immature seed (green pods). It is much easier to sterilize green capsules than individual seeds after the capsule has split open. Lucke (1971) indicated that orchid seed can be sterilized when the capsule is about two-thirds ripe.

Transfer of Explant

To minimize the risk of contamination during transfer of sterilized explant into the sterile culture vessel containing nutrient media, it is advisable that the operator should wear sterilized clothes and headgear, before entering the sterile area. The most important and easy precaution is to wash the hands with 70% ethyl alcohol before starting an experiment.Talking or sneezing during transfer or inoculation should be avoided. The cultured tissue should never touch inside the edges of the culture vessel. For aseptic manipulations in a tissue culture laboratory, certain precautions (Appendix 1) and steps (Appendix 2) have to be followed to carry out an experiment successfully.

Appendix 1.

Precautions Observed during Aseptic Manipulations in a Tissue Culture Laboratory

General

1. Enter the laboratory wearing clean shoes and laboratory coat.
2. Avoid handling alcohols around open flames.
3. Never pipette by mouth.
4. Handle strong acids and alkalis with extreme caution.
5. Wash (preferably with alcohol) and bandage all cuts immediately.
6. Ensure before opening an autoclave that the pressure is reduced to zero and the temperature is below 100°C.
7. Switch off the electrical appliances (e.g., stirrers, pH meters, balances, hood) when not in use, particularly overnight.
8. Cover all equipment to avoid contact with dust and other contaminants.
9. Clean benches and work surfaces regularly; take care that dirty glassware or other items are kept away while work is in progress.
10. Mop the floor in the laboratory and the culture room with an approved disinfectant.
11. Separate space should be allocated for storage of items such as chemicals, photographic equipment, cleaned labware and other solutions.

Appendix 2

Steps Involved in Aseptic in vitro Culture of Plant Tissues

1. Pieces of plant material are collected in a suitable container (screw-cap bottle) and immersed in a solution of the disinfectant at suitable concentrations.
2. The plant material is sterilised for the period given in Box 2.
3. During the period of sterilisation the container is shaken two to three

   times, the cap removed and the liquid poured out.
4. Care should be taken to recap the bottle before washing the plant material.

5. After washing, the surface-sterilised plant material is transferred to presterilised petri dishes (or china tile) and suitable explants prepared using sterilised instruments (scalpels, needles, cork-borer, forceps) and a dissection microscope.
6. The cap of the culture vial containing the nutrient medium (also presterilised) is removed, the inoculum (explant) transferred onto the medium and the culture vial recapped, all in quick succession. In the case of glass vial, the neck is flamed before closing it with the cap.
7. All operations are carried out in the laminar airflow hood.

Appendix 3

See Box 3. for an overall summary of the sterilization techniques:

Box 3.

Sterilization technique	Materials sterilized
Dry heat (160-180°C for 3 hr)	Empty glasswares (culture vessels, pipettes etc.), and certain plasticwares (Teflon FEP); instruments like scalpels, forceps, needles etc. (glass bead sterilizers used).
Flame sterilization	Instruments like scalpels, forceps etc.; mouths of culture vessels
Autoclaving (121° C at 15 p.s.i. for 15 – 40 min)	Media, culture vessels (glasswares and several plasticwares), other glass and plasticwares, contaminated cultures.
Filter sterilization *(a)* Liquid (membrane filter of 0.45 or smaller pore size)	Heat labile compounds like GA_3, ABA, Zeatin, Urea, enzymes etc.
(b) Air (HEPA filter)	Air blown through laminar flow cabinets
Wiping with 70% Ethanol	Platform of laminar flow cabinets, hands of the operator etc.
Surface sterilization (using one of several sterilizing agents)	All plant materials to be cultured

Conclusion

The maintenance of a completely aseptic environment inside the culture room and the culture vial where the tissue grows is absolutely essential. Since, microbes are omnipresent in our environment and on coming into contact with the culture media they find conditions optimal and grow much faster than the cultured tissue. Therefore, proper measures of sterilization as described in this Lesson must be followed in order to prevent the contamination of plant tissue cultures.

Questions

1. Describe the methods of sterilization under following heads:
   a. sterilization of culture room and transfer area
   b. sterilization of explant
   c. autoclaving.
2. Write down the steps involved in aseptic culture technique in vitro.

Note